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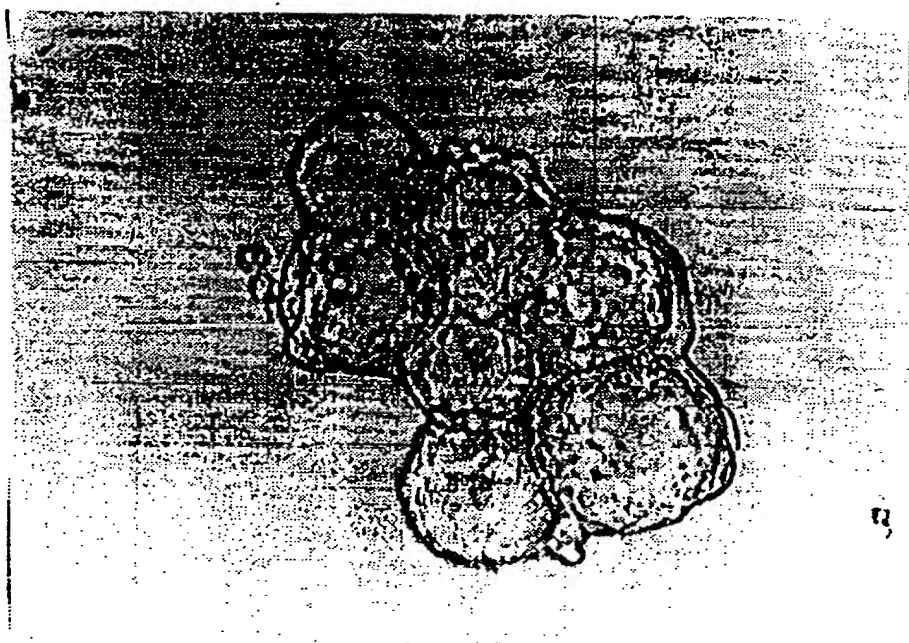
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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**(54) Title:** BIOMATERIAL CONTAINING EPITHELIAL CELLS AND USE THEREOF AS A TRANSPLANT

**(57) Abstract**

A biomaterial containing epithelial cells which are adherently attached to microcarriers is suitable for use in preparing a transplantation material for the treatment of wounds. The microcarriers preferably have a diameter of 50 to 500  $\mu\text{m}$  and a coverage with epithelial cells of 30 to 100 %.



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**Biomaterial containing epithelial cells and  
use thereof as a transplant**

The subject-matter of the invention is a new biomaterial which contains epithelial cells on microcarriers (MCs), as well as the preparation and use of said biomaterial for preparing a medicament.

Skin consists of the epidermis, the basement membrane and the dermis. The epidermis forms the outer coat. The dermis consists essentially of fibroblasts with additional substances such as, e.g., collagen. The basement membrane essentially contains keratinocytes which are non-differentiated but proliferating. The epidermis essentially contains differentiated keratinocytes wherein the upper layer is horny. It is true that while the degree of differentiation of the cells is increasing, the ability of the cells to proliferate is decreasing. Accordingly, the non-differentiated keratinocytes exhibit the highest proliferation potency.

Burn wounds and slow-healing wounds (ulcers) usually are treated with skin sheets consisting of autologous keratinocytes (classical method). To this end, a piece of skin is cut off from the patient, the keratinocytes are cultured in vitro, and the expanded skin sheets so obtained are transplanted [Gallico, G.G., et al., *New Engl. J. Med.* 311 (1984) 448-451 (1); Cuono, C., et al., *Lancet* (1986) 1123-1124 (2); De Luca, M., et al., *Burns* 15 (5) (1989) 303-309 (3); Gallico, G.G., *Clinics in Plastic Surgery* 17 (3) (1990) 519-526 (4); De Luca, M., and Cancedda, R., *Burns* 18 (1) (1992) 5-9 (5)]. The transfer of thin skin sheets from cell culture flasks to, e.g., Vaseline gauze as well as the fixing thereof onto the wound surface is problematical. The covering of wounds on parts of the body that are difficult to reach is another problem. Besides this, only keratinocytes can be transferred by means of such classical skin sheets. Skin sheets from keratinocytes involve the drawback that these cells are almost completely differentiated when the sheets are being formed, which means that the sheets practically no longer contain any keratinocytes capable of proliferation. Thus, these sheets constitute a transplantation material which cannot grow, or continue growing, after transplantation. Epidermal transplants (classical skin sheets) with allogenic keratinocytes have been carried out in a number of clinics without any immunological rejection reactions being observed (Gboyse, S.T., et al., *Plast. Reconst. Surg.* 91 (1993) 632 (17); Burt, A.M., et al., *Br. Med. J.* 298 (1989) 915 (18); Hickerson, W.L., et al., *Burns* 20/1 (1994) 52 (19)).

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From WO 92/06179 (6) and EP-A 0 242 270 (7) biomaterials are known which contain epithelial cells within a collagen gel. In this context, epithelial cells (fibroblasts or keratinocytes) are embedded in gel layers. It is known that such gels may also contain keratinocytes. However, the keratinocytes in the gel are immobilized and are thus incapable of proliferating and differentiating to an appreciable extent after transplantation. Accordingly, keratinocytes from such gels are not able to form a basement membrane and an epidermis. Another drawback of this biomaterial is, in particular, the low rate of migration of the epithelial cells from the biomaterial into the wound. Furthermore, the handling and fixing of these biomaterials is very difficult and laborious.

In WO 90/02796 (8) there is described a three-dimensional cell culture system on the basis of, for instance, cellulose, polyamide, polyester, on which epithelial cells can grow. In this context, an artificial tissue is obtained which is covered with epithelial cells (fibroblasts or keratinocytes) and which is said to be suitable for transplantation. These artificial transplantation materials, too, do not exhibit any advantage over the skin sheets applied in the classical method.

The object of the invention is to provide a biomaterial which does not exhibit the drawbacks described and which can be prepared in an easy manner and in large quantities.

The subject-matter of the invention is the use of a biomaterial which contains epithelial cells adherently attached to microcarriers, for preparing a transplantation material for the treatment of skin wounds. The present invention makes it possible to:

1. transfer non-differentiated and, thus, potent keratinocytes that are capable of proliferation;
2. form a basement membrane and an epidermis on the wound after transplantation (the non-differentiated keratinocytes, after transplantation, separate from the microcarriers and proliferate and differentiate).

It was heretofore unknown that non-differentiated keratinocytes which are capable of proliferation, can be cultured *in vitro*. The method according to the present invention, makes it possible to produce such keratinocytes. Thus, the present method is susceptible of industrial application directly *in vitro*. Non-differentiated keratinocytes produce components which are different from those produced by differentiated keratinocytes (which, according to the prior

art, have heretofore been the only ones obtained by culturing *in vitro*). An example of such a component is collagen IV. Accordingly, the invention offers a method of producing substances, such as collagen IV, by culturing non-differentiated keratinocytes. The non-differentiated keratinocytes can be selected by using immune staining with the markers Ki67, keratin 10 and keratin 14 (Slivka, S.R., et al., J. Invest. Dermatol. 100 (1993) 40-46 (15); Regnier, M., et al., Skin Pharmacol. 3 (1990) 70-85 (16)).

As epithelial cells, all epithelial cells, such as, for instance, keratinocytes, fibroblasts and endothelial cells, are suitable. Such cells preferably are proliferating cells, and most preferably, non-confluent cells which have the properties of basal cells. CellGenesys is engineering epidermal keratinocytes to have "universal properties" so that they can be used in a full thickness skin (epidermis and dermis) graft without causing a donor-host immune response. (CellGenesys, research collaboration with Advanced Tissue Sciences, SCRIP No. 1961, 27 Sept. 1994, p. 24).

By "microcarriers" there are to be understood small solid particles which are capable of being suspended in a slowly stirred, aqueous medium. Such microcarriers are used for the cultivation of eukaryotic cells, wherein the cells grow on the surface of these particles, in the form of a confluent layer. Microcarriers of this kind and their application in cell cultures are described, for instance, in Advances in Applied Microbiology 31 (1986) 139-179.

As microcarriers there may be used all microcarriers which consist of a biocompatible material (such as, e.g., collagen, polymers from hyaluronic acid or hyaluronane esters (e.g. ethyl or benzyl esters) (Andreassi, L., et al., Wounds 3 (1991) 116-126 (28); Myers, S.R., et al., Abstract No. 85 in 5th Annual Meeting of the European Tissue Repair Society (August 1995), Padua (29)) (e.g. HYAFF-11™ from FIDIA Advanced Biopolymers, Abano T., Italy), fibrin binding polypeptides, dextran, gelatin, silicone, etc.) or of a suitable mixture of several biocompatible materials. There may be used, for example, Cytodex3® (crosslinked dextran matrix with a denatured collagen layer, 100-230 µm) (Pharmacia AB, Sweden) based on dextran Cellgen (Koken Co., Japan), a reconstituted and crosslinked collagen from bovine skin, 200-500 µm, or Cultispher-G® (macroporous gelatin microcarrier) (Hyclone, Greiner, Germany) based on gelatin.

The size of the microcarriers is not critical. Usually, however, the carriers have a diameter of 50 to 500 µm. Microcarriers of a diameter below 50 µm are so small that they usually cannot be sufficiently covered with cells growing on them. Microcarriers of a size higher than 500 µm

are so large that, on the one hand, a high coverage with cells is difficult to achieve; and on the other hand, these particles are not optimal with regard to transplantations because the detachment of the epithelial cells and spreading on the wound does not proceed satisfactorily in the case of such particles.

Surprisingly, it has been found that for an epidermal graft according to the invention there need not be used autologous cells as are applied in the known methods, but rather allogenic cells alone or mixtures of autologous and allogenic cells are also suitable. This offers the advantage that it is not necessary to prepare for each patient, in a time-consuming cultivation, a completely autologous transplantation material. The allogenic transplantation material can be standardized and will be available then in large amounts and at any time. This is especially important in the case of use for the treatment of large-area burns which must be treated as quickly as possible.

Allogenic keratinocytes, after transplantation, are essentially not rejected. However, it is not these keratinocytes themselves which form the new skin. The essential function of the non-differentiated allogenic keratinocytes is to stimulate those keratinocytes of the transplant recipient which are still present (these non-differentiated keratinocytes will form other components than those formed by differentiated keratinocytes). They secrete a large number of hormones (vitamin D, steroids, and others), cytokines (interleukin 1, 3, 6, PDGF, bFGF, TGF- $\alpha$ , TGF- $\beta$ , and others) as well as matrix elements (laminine, type IV/-VII collagen, fibronectin), which effect an accelerated epithelization extending from the margin of the wound as well as of remaining areas of cutaneous appendage (which still contain residues of intact keratinocytes) (Brown, H., "Wound healing research through the ages", wound healing: biochemical and clinical aspects, Saunders, Philadelphia, page 5, editors: Cohen, I.K. Diegelman, R.F., and Lindblad, W.J., 1992 (14)). In burns of the IIb degree, for instance, where residues of the patient's own keratinocytes are still present, using an allogenic transplant or a mixed transplant comprising allogenic and autologous keratinocytes, will be sufficient. The allogenic keratinocytes stimulate the remaining keratinocytes of the recipient and themselves die off after some time. In high degree burns (third degree), practically no keratinocytes will be present any longer. In such cases, a transplantation of autologous keratinocytes is necessary.

The time until the start of treatment can be considerably shortened also by using a biomaterial containing a reduced portion of autologous cells. In a preferred embodiment, the biomaterial may contain 10 to 30% of autologous cells. As far as the remainder of 70 to 90% is



concerned, one may resort to stored allogenic biomaterial available in large amounts. By this the time for preparing a sufficient amount of transplantation material can be shortened because only a small amount of autologous cells of the cultivation need be obtained.

In addition to this, the biomaterial according to the invention offers new possibilities of preparing individual transplantation materials. For example, it is possible to prepare the transplantation material immediately before transplantation. This can also be done by mixing biomaterial according to the invention containing autologous and/or allogenic epithelial cells and/or various epithelial cells (e.g. keratinocytes or fibroblasts). By this the specific requirements of the wound to be treated (e.g. degree of burn, burn wound or type of wound) can be taken into account optimally.

This individual adaptation of the composition of the biomaterial to the transplantation problem is impossible to achieve with the prior art transplantation materials.

In order to prepare the biomaterial, the autologous and/or allogenic epithelial cells are isolated, in a manner known to one skilled in the art [Rheinwald, J.G., and Green, H., *Cell* 6 (1975) 331-334 (9); Limat, A., et al., *J. Invest. Dermatol.* 92 (1989) 758-762 (10)], from human or animal material and, suitably, are first pre-cultured adherently. Preferably after the first or the second passage, the cells are cultured further together with the microcarriers. This is accomplished either in a stirred culture (spinner culture or fermenter) or in a quiescent culture (e.g. cell culture flasks), wherein the cells spread out from the bottom of the cell culture flasks onto the microcarriers. It is also preferred to incubate beads, which have been allowed to swell in water, with the cells over a period of 2 to 5 hours, preferably 3 to 4 hours, and while doing so, to stir the culture briefly (for 3 to 5 minutes) once an hour.

In a preferred embodiment, the microcarriers can be pre-coated in order to improve the adhesion of the cells and enhance the extent of coverage of the microcarriers. For the coating, for instance proteins of the extracellular matrix, such as fibronectin or collagen, or whole eukaryotic cells (e.g. fibroblasts incapable of proliferation) or components thereof (e.g. membrane fractions) may be used.

Preferably, prior to cultivation, the microcarriers are coated with epithelial cells incapable of proliferation, such as, e.g., fibroblasts. Suitable fibroblasts are, for example, killed mouse fibroblasts from the cell line 3T3. Such a pre-coating according to the methods known to one skilled in the art is described in DE 26 51 685, for instance. It is also preferred to pre-coat the

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microcarriers with substances which improve the adhesion of the cells (e.g. collagen). Suitably, the pre-coating is carried out after swelling of the microcarriers and autoclaving for sterilization, but before incubation with the epithelial cells. To this end, the microcarriers are incubated with an aqueous solution of such an adhesion molecule or the cell suspension (the latter, suitably, in cell culture medium). Thereafter, the coated microcarriers are washed [preferably with PBS (phosphate buffered saline) NaCl: 8000 mg/l, KCl: 200 mg/l, Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O: 1440 mg/l, KH<sub>2</sub>PO<sub>4</sub>: 200 mg/l].

In order to coat the microcarriers with the epithelial cells, preferably the cultivation is carried out in serum-free medium, optionally with pituitary extract (bovine) or fetal calf serum being added.

In a preferred embodiment, growth- and cell differentiation-promoting factors, respectively, such as keratinocyte growth factor (KGF), nerve growth factor (NGF) or platelet-derived growth factor (PDGF), are added in order to enhance the extent of coverage.

After the desired extent of coverage has been attained, the cells are isolated. This can be accomplished, for instance, by sedimentation or centrifugation at low g-values (e.g. 10 to 50 g).

The cells isolated in this manner either are applied directly in a suitable galenic formulation for treatment of wounds or are preserved until treatment. For preservation, ordinarily cryopreservation is suitable. Cryopreservation is carried out preferably with the addition of cryoprotective substances, such as dimethyl sulfoxide (DMSO) or glycerol, at a temperature of +3°C to -196°C, preferably for long-term storage (more than 6 hours). In the case of short-term storage (less than 6 hours), preservation can be carried out at a temperature of +3°C to +10°C without the addition of cryoprotective substances.

In order to prepare a biomaterial containing autologous and allogenic cells attached to microcarriers, preferably autologous epidermal cells are first isolated and cultured in the presence of the microcarriers. The cultivation is carried out for a sufficient time until the amount of autologous biomaterial desired as a proportion in the mixture has been attained. Then, allogenic biomaterial (which contains allogenic epithelial cells adherently attached to the microcarriers) is added and the epidermal graft according to the invention is prepared, as a mixture. In this context, it is preferred to employ cryopreserved allogenic biomaterial or allogenic biomaterial that has been stored at a temperature between +3°C and +10°C.

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Preferably, the proportion of autologous biomaterial in the mixture is between 10 and 30%. It is also possible to culture autologous and allogenic cells together in the presence of microcarriers. In such case, one will obtain a transplantation material in which both autologous and allogenic cells are attached to the individual microcarriers.

In a further preferred embodiment, a mixture is used which consists of microcarriers wherein each individual microcarrier particle is covered with one type of epithelial cells only, such as, e.g., fibroblasts or keratinocytes. It is also possible to use microcarriers on which several types of epithelial cells (preferably, fibroblasts and keratinocytes (fetal or adult)) are adherently attached. The ratio of mixing of the differently covered microcarriers or the ratio of mixing of the individual cells on one microcarrier may actually be any ratio desired and may vary depending on the intended use (e.g. burn wounds, senile ulcers, diabetic ulcers). In this context it is preferred to apply, for diabetic wounds, only fibroblasts, or fibroblasts in excess, and for burn wounds, only keratinocytes, or keratinocytes in excess over the other epithelial cells in the transplantation material.

The extent of coverage of the microcarriers with epithelial cells can be varied widely. However, the lower the coverage, the lower the efficacy of the transplant. Hence, in practice, it is advantageous to apply a coverage as high as possible.

The preferred coverage is between 30 and 100%. Surprisingly, it has been found, however, that a coverage between 50 and 80%, preferably between 60 and 70%, is optimum.

By coverage there is to be understood the average amount of cells adherently attached to the microcarrier, in reference to complete coverage.

This amount can be determined either visually under the microscope or after detachment of the cells from the microcarriers (for instance, by treatment with trypsin) and staining. Suitably, the complete coverage can be determined most easily visually under the microscope. Typical pictures of a 30% and 80% coverage of the microcarriers according to the invention are shown in Figures 1 and 2.

For therapeutic application, the preserved biomaterial (optionally mixed with fresh biomaterial which may also be autologous) is used.

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To this end, the biomaterial is thawed, optionally the cryoprotective substance is removed by washing with medium, and the biomaterial is mixed with a preparation which constitutes the galenic medium. It is essential for the galenic formulation that the material used should not dehydrate the cells. It has been found that hydrogels, preferably based on methyl cellulose, are particularly suitable for this purpose. Examples of suitable hydrogels are as follows:

— methyl cellulose (preferably about 4%, non-ionogenic cellulose derivatives, i.e., modified D-glucoglycans in which the hydroxyl groups of the cellulose are substituted with methyl groups) (Keresztes, A., Kedvessy, G., *Pharmacia* 20 (1965) 371 (20); Sarkar, N., *J. Appl. Polymer Sci.* 24 (1979) 1073 (21);

— alginates (salts of alginic acid, e.g., sodium-, calcium-, magnesium- or ammonium alginate, e.g., Kelco-Alginates® of the firm of Kelco) (Sellerie, R., et al., *Boll. chim. farmaceut. Milano* 119 (1980) 41 (22); Daigo, K., et al., *Yakuri to Chiryo* 11 (1983) 401 (23);

— agarose (polysaccharide from agarobiose and neo-agarobiose) (Langford, J.J., Dambach, A.E., *Soap. Chem. Specialties* 45/5 (1969) 231 (24).

Type I collagen and type III collagen in medium may also be suitable.

It is also possible to use methyl cellulose dissolved in culture medium. The viscosity must be such that the material is easy to spread and that the cells can separate after transplantation and are capable of migration in order to form, e.g., as keratinocytes a basement membrane and an epidermis.

The basis of the galenic medium suitably is essentially the culture medium. It is, however, advantageous to add further substances which are compatible with the skin and with which the viscosity of the biomaterial can be altered. Optimizing the viscosity is especially advantageous for optimizing the handling of the biomaterial in applying it to the wounds, and for adhesion to the wounds. Suitable skin-compatible substances for altering the degree of viscosity are, for instance, thickening agents, auxiliary emulsifying agents, hydrocolloids or gelling agents, such as, e.g., gelatin, pectin, alginates, cellulose derivatives, Aqualose and polysaccharides.

Suitably, these substances are admixed to the galenic medium at such concentration that the microcarrier preparation is easy to apply.

In a preferred embodiment, the galenic medium may further contain substances that additionally improve wound healing, such as, e.g., NGF, TGF- $\beta$ , PDGF, KGF and/or fibronectin or analogs (Schärfetter-Kochanek, K., et al., J. Invest. Dermatol. 98 (1992) 3-11 (30); Lange, T.S., et al., J. Exp. Dermatol. 4 (1995) 130-137 (31); WO 92/09200 (32) (for fibronectin peptides); WO 90/07577 (33); WO 91/17765 (36)), which improve the adhesion of the epithelial cells on the wound bed, especially after the cells have become detached from the carrier. Preferably, the medicament contains between 0.03 g and 0.1 g of fibronectin or analogs per gram of galenic medium. It is further preferred that the galenic medium should contain viable cells having the properties of macrophages, e.g., macrophages or Langerhans' cells for disinfecting the wounds. It is preferred that the medicament should contain about 10 to 30% of such cells.

The medicament according to the invention, as an epidermal graft, is suitable for use in the treatment of, for instance, burn wounds, diabetic wounds, senile ulcers and other slow-healing wounds. The medicament according to the invention is also suitable for speeding up the healing of normal wounds. A further use is in plastic surgery.

An essential advantage of the biomaterial according to the invention lies in the fact that, unlike the known epidermal grafts, it is flexible in all directions and easy to adapt to the body surface even on parts which are problematical (e.g. skinfolds, ear, eyelids).

For application, suitably, the medicament according to the invention is placed on the wounds using an appropriate dressing depending on the type of wound to be treated [for example: a) fatty gauze dressing, e.g., GRASSOLIN® of the firm of Hartmann, Germany, b) hydrophobic polymer dressing, e.g., EPI-LOCK® of the firm of Merck & Co., Missouri/USA, c) hydroactive dressing, e.g., DUODERM® [gel formula dressing] of the firm of Conva Tec, Princeton/USA d) MITRAFLEX (microporous polyurethane membrane)], in order to maintain its effectiveness.

In a preferred embodiment, a mixture of autologous and allogenic epithelial cells, in particular keratinocytes, is used.

As keratinocytes, preferably epidermal keratinocytes (e.g. foreskin keratinocytes) are used.

In order to prepare a biomaterial based, in particular, on keratinocytes it is specifically preferred to pre-coat the microcarriers, as described above. Intermittent stirring is also

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preferred in the culturing of the cells in the presence of the microcarriers. To this end, the culture is preferably stirred for a few minutes (e.g. 1 to 5 minutes) at a speed between 10 and 100 rpm and subsequently cultured further for an extended period of time (about 20 to 40 minutes), without stirring. The intermittent stirring conditions are maintained over the entire period of culturing. It has been found that it is specifically advantageous for the culturing if stirring is carried out for about 2 minutes at about 40 rpm, followed by a rest period of about 30 minutes. These conditions may, of course, be varied to a certain extent. In this context care must be taken that stirring is carried out for a period as short as possible, yet attaining a thorough mixing of the medium, so that subsequently, culturing can be carried out as long as possible without stirring and with the cell growth and the adhesion of the cells to the microcarriers being impaired as little as possible.

In order to achieve a high coverage of the microcarriers in culturing, in particular, keratinocytes, it is further preferred to carry out the culturing first, in a first culturing phase, in an amount of culture medium as low as possible. This amount must be just high enough to ensure that the cells do not die to a considerable extent and are capable, at the same time, of binding to the surface of the microcarriers. It is only after the keratinocytes are adherently attached to the microcarriers to a considerable extent (usually after 6 to 8 hours) that, in a second culturing phase, further medium is added. The total amount of medium corresponds to that amount of medium which is optimally applied for the coverage of the microcarriers using uncritically growing epithelial cells, such as fibroblasts. For microcarriers which are known as carriers for adherent cells, this amount is usually specified by the manufacturer. In this context, the optimum amount of medium is 100 ml for 300 mg of Cytodex® (crosslinked dextran matrix) and for 100 mg of Cultispher® (macroporous gelatin microcarrier). For culturing the keratinocytes, in the first culturing stage, only about 40 to 60% of this optimum volume of cell culture medium is added.

The examples, publications and figures provided below shall illustrate the invention further, the protective scope of which is established by the patent claims. The methods described are to be understood as examples which describe the subject matter of the invention, even after modifications have been made.

**Fig. 1** depicts Cytodex 3® (crosslinked dextran matrix with a denatured collagen layer) microcarriers with a 30% coverage with keratinocytes (example 2.2).

**Fig. 2** depicts the same system with an 80% coverage.

**Fig. 3** depicts the migration of keratinocytes on the artificial dermis (Example 5).

### **Example 1**

#### **Isolation and cultivation of human keratinocytes**

##### **1) Epidermal keratinocytes**

Epidermal cells (keratinocytes) are isolated enzymatically from pieces of skin and foreskin, respectively, and are placed in the cell culture.

Isolation is carried out according to methods known in the art [Rheinwald, J.G., and Green, H., *Cell* 6 (1975) 331 - 334 (9); Limat, A., et al., *J. Invest. Dermatol.* 92 (1989) 758 - 762 (10); Rheinwald, J.G., *Methods Cell. Biol.* 21A (1980) 229-254 (12)].

The cultivation and proliferation of the keratinocytes until use for coverage of the microcarriers (MCs) is carried out in a static culture (cell culture flasks or cell factories) with serum-free medium.

##### **2) Cells of the outer root sheath (ORS keratinocytes) from hair follicles**

The establishment of primary cultures of ORS cells and expansion (cultivation) thereof in vitro is accomplished according to methods known in the art [Limat, A., et al., *J. Invest. Dermatol.* 92 (1989) 758 - 762 (10); Limat, A., and Noser, F., *J. Invest. Dermatol.* 87 (1986) 485-488 (13)].

### **Example 2**

#### **Preparation of the microcarriers and cultivation of keratinocytes on microcarriers (MCs) (coverage of the MCs)**

##### **2.1 Preparation of the MCs for the cultivation**

###### **a) Cytodex 3® (Pharmacia - crosslinked dextran matrix with a denatured collagen layer)**

The MCs are weighed and allowed to swell in phosphate-buffered saline without calcium ions and magnesium ions (PBS -/-) overnight at room temperature (to be stirred up occasionally at

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the beginning). The PBS is decanted and the MCs are additionally washed twice with PBS; 50 to 100 ml PBS per gram of MC are applied.

The suspension is autoclaved (115°C, 15 psi, 15 min.) and thereafter the MCs are placed in culture medium (GIBCO, serum-free Keratinocyte-SFM). The MCs are washed once with culture medium and kept in the same medium at 4°C.

b) **Cultispher®** (Hyclone - macroporous gelatin microcarrier)

The MCs are weighed and allowed to swell in phosphate-buffered saline without calcium ions and magnesium ions (PBS -/-) overnight at room temperature (to be stirred up occasionally at the beginning). The PBS is decanted and the MCs are additionally washed twice with PBS; 50 to 100 ml PBS per gram of MC are applied.

**Pre-coating with collagen**

The suspension is autoclaved (115°C, 15 psi, 15 min.). Subsequently, the MCs are stirred overnight at 37°C in a solution of collagen S type I (3 mg collagen in 10 ml of 0.1% CH<sub>3</sub>COOH); 10 ml collagen solution per 100 mg MC are applied. The supernatant is discarded, the MCs are washed once with PBS-/- and placed in culture medium (GIBCO, serum-free keratinocyte-SFM). After single washing (with culture medium), the MCs are kept in culture medium at 4°C.

## **2.2 Cultivation of the keratinocytes on the MCs**

Subconfluent (about 70%) serum-free cultured keratinocytes (in cell culture flasks) of the first or the second passage are released from the bottom of the culture vessel using a trypsin/EDTA solution (5 ml per cell culture flask [175 cm<sup>2</sup> culture area] of a 0.05% trypsin/0.02% EDTA solution in PBS-/-, period of action: about 5 min. at 37°C). The release through trypsinization is stopped with 3 ml FCS (fetal calf serum). This suspension of individual cells is removed by centrifugation (10 min., at 180 g). The cell pellet is thoroughly resuspended in culture medium and cell counting is carried out using trypan blue.

Seeding with the MCs is carried out in a spinner flask (effective volume 50 ml). To about 1 x 10<sup>7</sup> cells are added 300 mg Cytodex 3-MC and 100 mg Cultispher respectively (about 10 cells per carrier) and the liquid volume is adjusted to 30 ml with serum-free culture medium.



Stirring in the spinner flask is carried out intermittently: 2 min. at 40 rpm, followed by 30 minutes of cultivation without stirring. These stirring conditions are maintained during the whole period of cultivation.

After 6 to 8 hours, medium is added until a volume of 100 ml is attained (possibly, the culture is transferred to a spinner flask having an effective volume of 250 ml). During the further period of cultivation, the medium is changed twice a day (about every 12 hours) according to the following procedure: The carriers settle on the bottom of the flask, spent medium is sucked off to the highest possible extent and replaced by fresh medium.

During the period of cultivation, the coverage of the MCs is evaluated microscopically and documented photographically. In the case of the Cytodex 3 MCs, the cells are evaluated without staining. In the case of the Cultispher MCs, the keratinocytes are stained with the Hoechst dye 33342 (2'-[4-Ethoxyphenyl]-5-[4-1-piperazinyl]-2,5'-bi-1H-benzimidazole) beforehand. Examples using cells on Cytodex 3 MCs:

**Figure 1:** About 30% coverage.

**Figure 2:** About 80% coverage.

After the desired coverage has been attained, excess medium (after the settling of the carriers) is sucked off and the remaining suspension is removed by centrifugation at 10 to 50 g for 10 min. A galenic substance is admixed to the pellet and the whole thing is applied in this form, or the pellet is frozen with the addition of a cryoprotectant for use at a later date. The table given below shows the extent of keratinocyte coverage of the microcarriers in dependence upon the optimizing process features:

- intermittent stirring
- increasing the volume of the medium after 6 to 8 hours

Volume	Stirring	Coverage
constant	continuously	0
constant	intermittently	~20%
increase 50%	continuously	~20%
increase 50%	intermittently	~80%

It can be seen from Table 1 that each of these optimizing features contributes to enhancing the coverage. The combination of intermittent stirring and increase in volume leads to a decisive improvement of the coverage.

### 2.3 Fast cultivation of keratinocytes (Kz) on MCs

The MCs are incubated at a concentration of  $1.2 \times 10^5$  MC/ml of culture medium with keratinocytes (cultured serum-free or with serum) at a concentration of  $1 \times 10^6$  Kz/ml of culture medium. The ratio of MC : Kz is at least 1 : 10. The incubation takes place in a 13 ml centrifuge tube in an incubation cabinet (37°C, 5% CO<sub>2</sub>, 95% rH) for at least 2 hours, not more than 6 hours, preferably for 3 hours. The suspension is to be shaken carefully once an hour for about 20 seconds. After that period, the majority of the Kz have adhered to the MC.

After 3 to 4 hours an aliquot is taken from the suspension and the coverage is examined microscopically.

MC	Number of covered MCs, in %	Coverage density of the individual MCs, in %
Cytodex 3	50 - 70	70 - 90
Cellgen	70 - 90	70 - 90
Biosilon	50 - 70	40 - 70
Cultispher	0 - 5	0 - 5
Collasphere	0 - 5	0 - 5

#### Example 3

##### Galenic medium

Keratinocytes alone (without beads) were mixed with several substances (but always with medium) and examined for viability. The results are as follows:

- in gelatin: low viability;
- in a number of ready-to-use wound gels/ointments: low or poor viability
- in Nifucin-Gel® of the firm of Apogepha (nitrofurazone and adjuvant): good viability.

**Example 4****Determination of the coverage of the microcarriers:**

The coverage of the microcarriers (determining the number of cells) is determined by means of protein determination. The protein determination is carried out according to the BCA method (test kit of the firm of Pierce, Catalogue No.: 23225).

**Preparing the samples:**

1 ml of a homogeneous suspension (microcarriers covered with cells in medium) is transferred to an Eppendorf vessel and removed by centrifugation in the Eppendorf centrifuge (2 min. at 10,000 rpm). The supernatant is discarded. The pellet is resuspended in 200 µl CAT lysis buffer (containing MOPS, NaCl and Triton X-100, pH 6.5, product of the firm of Boehringer Mannheim GmbH from CAT-ELISA-Kit, Catalogue No.: 1363727), resuspended and incubated at room temperature for 1 h.

**Preparing the standards (standard series):**

Keratinocytes from a confluent static culture are brought into suspension using a trypsin EDTA solution (0.05% trypsin/0.02% EDTA in PBS (Ca/MG-free). After the cell count (trypan blue method), 5 samples comprising in each case  $1 \times 10^5$ ,  $2.5 \times 10^5$ ,  $5 \times 10^5$ ,  $1 \times 10^6$ ,  $2 \times 10^6$  cells are prepared from this cell suspension. The samples are removed by centrifugation (Eppendorf centrifuge, 2 min. at 10,000 rpm) and the pellets are resuspended in each case in 200 µl CAT lysis buffer (see above) and incubated at room temperature for 1 h. Subsequently, the protein determination of the standards and the samples is carried out according to the BCA method (see above). The OD values of the standards (x axis) are plotted in a diagram against the cell counts in the standards (y axis). The OD values of the samples are read with the help of this standard curve in cell count/ml.

**Calculating the extent of coverage:**

- a) According to theoretical calculations, the microcarriers, at a coverage of 100%, carry, on an average, 40 keratinocytes/microcarrier.

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- b) The number of microcarriers employed in the samples is known:

$4 \times 10^4$  microcarriers/ml sample.

- c) The maximum possible number of cells in the sample (at a 100% coverage of the microcarriers) is:  $1.6 \times 10^6$  keratinocytes/ml sample
- d) The cell count/ml in the samples read from the standard curve (protein determination) is compared with the maximum possible number of cells (see above: c) and, from this, the extent of coverage of the microcarriers with keratinocytes, in percent, is calculated.

#### **Example 5**

#### **in vivo assay**

The migration of the keratinocytes from the MCs was examined on a slightly modified "Air-Liquid-Interface" model (Noser, F.K., and Limat, A., *In Vitro Cell & Dev. Biol.* 23 (8) (1987) 541-545 (34)). Collagen gels having inactivated fibroblasts embedded therein were used as the artificial dermis (Bell, E., et al., *Proc. Natl. Acad. Sci. USA* 76 (1979) 1274-1278 (35)). Into these gels in each case 200  $\mu$ l of a MC keratinocyte suspension were seeded and cultivated, in a submersed manner, with a FCS-containing culture medium for a period of 24 to 48 hours. Subsequently, the gels were placed on a fine Teflon net and the same was disposed on a Teflon ring. This arrangement was placed in Petri dishes and cultivated for a period of up to three weeks. The serum-containing culture medium was exchanged completely every other day and the volume was measured such that it extended as far as to the lower edge of the Teflon net. For carrying out the histological or immunohistochemical examinations, the gels were lifted off from the Teflon net and were either fixed in a 4%-formalin solution and embedded in paraffin or subjected to cryoconservation. The histological sections of these preparations were dyed histologically with haematoxylin and eosin, or immunohistochemically with antibodies against CK-5, CK-10, mip (Ki67), and collagen IV. It was shown that migration of the keratinocytes takes place from the MCs onto the artificial dermis (Figure 3), that the development of stratified dermis occurs, and that keratinocytes having basal properties are present in the preparation.

**Example 6****In vivo tests**

Swine is the animal species suitable for the in vivo tests (Kangesu, T., et al., Br. J. Plastic Surgery 46 (1993) 401-409 (25); de Vries, H., et al., Wound Rep. Reg. 1 (1993) 244-252 (26); Breuing, K., et al., J. Surg. Res. 52 (1992) 50-58 (27)).

- MC : microcarriers without any coating  
MCG : carrier gel with 4% methyl cellulose (without any microcarriers)  
Kzy + MC : microcarriers (beads) coated with keratinocytes

The skin wounds are divided into five groups (group 1: control, without any applied substance; group 2: MC; group 3: MCG; group 4: MC + MCG; group 5: MC + MCG + Kzy). In order to be able to evaluate the tissue reactions occurring in each group, a number of seven preparations must be evaluated histologically. As the time course of the reaction is also important, the animals are examined after 3, 7 and 14 days. The points in time 3 and 7 days are covered by biopsies whilst after 14 days, the animals are sacrificed. Since as much as 30 skin wounds can be inflicted on each animal ((25), (26), (27)) there results a number of animals of  $n = 5$ . On each animal 23 (3 times 7 + 2 reserves) skin wounds are inflicted.

**Anesthesia:**

Sedation with Ketamin/Azaperon im; initial anesthesia with Propofol iv; prolonged anesthesia: Isofluran, oxygen, nitrous oxide (intubation).

**Operation:**

Under absolutely sterile conditions, the skin areas are cleaned, freed from fat and disinfected, skin wounds of standardized sizes and depths are inflicted (with a pattern and a dermatome; size: 1.5 x 1.5 cm; depth: 3 mm), the substances to be tested are applied on the wounds, the individual wounds are covered by watertight and airtight vinyl chambers (these chambers allow separate observation of the individual wounds; such wound chambers are also used in clinics, for the covering of skin wounds; manufacturer: P.A. Medical Co., Columbia, USA).

**Postoperative pain therapy:**

As the skin wounds located on the backs of the animals, and the wound chambers placed thereon, cause pain and/or feelings of discomfort (e.g. itching) in the animals at least in the

first postoperative phase, there is carried out routinely a therapy with Metamizol for a period of 3 days.

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**Patent Claims**

1. A transplantation material for the treatment of skin wounds, comprising a biomaterial containing epithelial cells attached to microcarriers.
2. The transplantation material according to claim 1, wherein said epithelial cells are selected from the group consisting of autologous cells, allogenic cells or a combination of autologous cells and allogenic cells.
3. The transplantation material according to claim 2, wherein said epithelial cells are a combination of 10-30% autologous cells and 70-90% allogenic cells.
4. The transplantation material according to claim 1, wherein said epithelial cells are selected from the group consisting of keratinocytes, fibroblasts, endothelial cells and any combination thereof.
5. The transplantation material according to claim 1, wherein said microcarriers are biocompatible materials.
6. The transplantation material according to claim 1, wherein said biocompatible materials are selected from the group consisting of dextran, gelatin and silicon.
7. The transplantation material according to claim 1, wherein said microcarriers have a diameter of 50 to 500  $\mu\text{m}$ .
8. The transplantation material according to claim 1, wherein said microcarriers exhibit an average coverage between 30 and 100% of the maximum coverage possible with epithelial cells.
9. The transplantation material according to claim 1, wherein said microcarriers are pre-coated with a coating selected from the group consisting of eukaryotic cells or components thereof and extracellular matrix proteins before said epithelial cells are attached.
10. The transplantation material according to claim 9, wherein said extracellular matrix proteins are selected from the group consisting of fibronectin and collagen.



11. The transplantation material according to claim 1, wherein said biomaterial is stored prior to use at a temperature of +3°C to +10°C.
12. The transplantation material according to claim 1, further comprising the addition of cryoprotective substances to said biomaterial, and storage of said biomaterial at a temperature between +3°C to -196°C prior to use.
13. The transplantation material according to claim 12, wherein said cryoprotective substances are selected from the group consisting of dimethyl sulfoxide and glycerol.
14. The transplantation material according to claim 1, wherein said biomaterial is a mixture of stored and freshly prepared biomaterial.
15. The transplantation material according to claim 1, wherein said biomaterial comprises keratinocytes attached to microcarriers with a coverage of more than 30%.
16. The transplantation material according to claim 1, wherein said wound is a diabetic wound and said epithelial cells are fibroblasts.
17. The transplantation material according to claim 1, wherein said wound is a burn wound and said epithelial cells are keratinocytes.
18. The transplantation material according to claim 1, wherein said epithelial cells are allogenic cells.
19. A method for preparing a biomaterial, comprising the steps of:
  - pre-culturing epithelial cells,
  - then culturing the cells in the presence of microcarriers to produce cell covered microcarriers, and
  - isolating any cell-covered microcarriers when a coverage of 30 to 100% is attained.
20. The method according to claim 19, further comprising intermittently stirring said cells during the culturing of said cells in the presence of microcarriers.

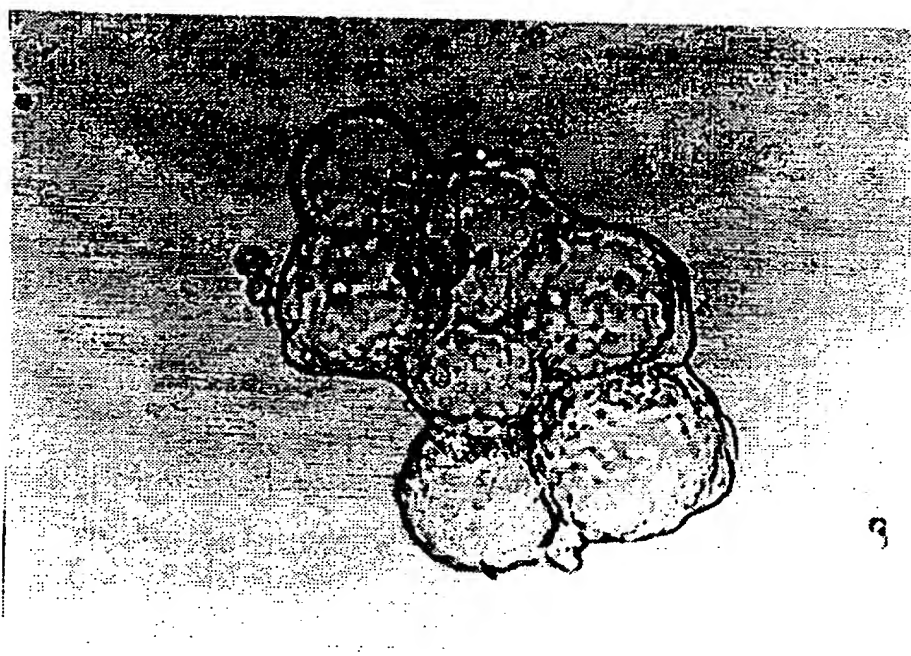
21. The method according to claim 20, wherein said cells are stirred for 2 minutes at 40 rpm then cultured for 30 minutes without stirring.
22. The method according to claim 19, wherein said culturing of the cells in the presence of microcarriers is initially carried out in less than an optimum volume of cell culture medium and an amount of cell culture medium is added to adjust the volume to the optimum volume after said cells are attached to said microcarriers.
23. The method according to claim 22, wherein said amount of cell culture medium is adjusted to the optimum volume after 6 to 8 hours.
24. The method according to claim 19, further comprising precoating said microcarriers with a coating selected from the group consisting of eukaryotic cells or components thereof and extracellular matrix proteins before culturing said cells in the presence of said microcarriers.
25. The method according to claim 19, wherein said cells are cultured in serum-free medium.
26. The method according to claim 19, further comprising the addition of pituitary extract or fetal calf serum to the medium.
27. The method according to claim 19, further comprising the addition of growth-promoting or cell differentiation-promoting factors to improve said coverage.
28. The method according to claim 27, wherein said growth-promoting or cell differentiation-promoting factors are selected from the group consisting of keratinocyte growth factor, nerve growth factor and platelet-derived growth factor.
29. The method according to claim 19, further comprising the addition of a suitable galenic formulation to said isolated cell-covered microcarriers.

30. A method for treating a skin wound comprising the steps of:  
  
applying a biomaterial comprising epithelial cells attached to microcarriers to said skin wound, and  
then covering said skin wound with a protective dressing.
31. The method according to claim 30, wherein said epithelial cells are selected from the group consisting of autologous cells, allogenic cells or a combination of autologous cells and allogenic cells.
32. The method according to claim 30, wherein said skin wound is selected from the group consisting of burn wounds, diabetic wounds, and senile ulcers.
33. The method according to claim 30, wherein said epithelial cells are allogenic cells.
34. A kit for treating a skin wound comprising a biomaterial containing epithelial cells attached to microcarriers and a protective wound dressing.
35. The kit according to claim 34, wherein said dressing is selected from the group consisting of Grassolin, Mitraflex, Duoderm and Epi-lock.
36. The kit according to claim 34, wherein said epithelial cells are allogenic cells.

Figure 1



Figure 2



**Figure 3**



# INTERNATIONAL SEARCH REPORT

International Application No.

PC/EP 95/04164

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 A61L27/00 C12N5/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 A61L C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A.93 23088 (CANADA NAT RES COUNCIL ;ARMSTRONG DAVID W (CA)) 25 November 1993 see page 8, line 28 - line 35 see page 19, line 2 - line 15; claims ---	1-36
X	DATABASE WPI Section Ch, Week 9436 Derwent Publications Ltd., London, GB; Class B04, AN 94-292256 & RU,C,2 010 028 ( MED BIOTECHN RES PRODN CENTRE) , 30 March 1994 see abstract ---	1-5
A	WO,A.90 13625 (DANOCHEMO AS) 15 November 1990 see claims ---	1-36
-/--		

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
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Date of the actual completion of the international search

16 February 1996

Date of mailing of the international search report

5 March 1996 (5.03.96)

Name and mailing address of the ISA

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## INTERNATIONAL SEARCH REPORT

International Application No

PC1/EP 95/04164

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 489 332 (BOEHRINGER INGELHEIM KG ;BOEHRINGER INGELHEIM INT (DE)) 10 June 1992 see claims ---	1-36
A	GB,A,2 059 991 (PHARMACIA FINE CHEMICALS AB) 29 April 1981 see the whole document -----	1-5



# INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 95/04164

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:

**Remark :** Although claims 1-18, 30-32 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PC 1, EP 95/04164

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9323088	25-11-93	AU-B- 4257993	13-12-93
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		SE-A- 7907573	13-03-81
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# INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen

PC./EP 95/04164

A. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES  
IPK 6 A61L27/00 C12N5/00

Nach der Internationalen Patentklassifikation (IPK) oder nach der nationalen Klassifikation und der IPK

## B. RECHERCHIERTE GEBIETE

Recherchierte Mindestprüfstoff (Klassifikationssystem und Klassifikationssymbole)

IPK 6 A61L C12N

Recherchierte aber nicht zum Mindestprüfstoff gehorende Veröffentlichungen, soweit diese unter die recherchierten Gebiete fallen

Während der internationalen Recherche konsultierte elektronische Datenbank (Name der Datenbank und evtl. verwendete Suchbegriffe)

## C. ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
X	WO,A,93 23088 (CANADA NAT RES COUNCIL ;ARMSTRONG DAVID W (CA)) 25.November 1993 siehe Seite 8, Zeile 28 - Zeile 35 siehe Seite 19, Zeile 2 - Zeile 15; Ansprüche ---	1-36
X	DATABASE WPI Section Ch, Week 9436 Derwent Publications Ltd., London, GB; Class B04, AN 94-292256 & RU,C,2 010 028 ( MED BIOTECHN RES PRODN CENTRE) , 30.März 1994 siehe Zusammenfassung ---	1-5
A	WO,A,90 13625 (DANOCHEMO AS) 15.November 1990 siehe Ansprüche ---	1-36
	--- -/-	

☒ Weitere Veröffentlichungen sind der Fortsetzung von Feld C zu entnehmen

☒ Siehe Anhang Patentfamilie

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\*Z\* Veröffentlichung, die Mitglied derselben Patentfamilie ist

Datum des Abschlusses der internationalen Recherche

16. Februar 1996

Absenddatum des internationalen Recherchenberichts

5 März 1996 (5.03.96)

Name und Postanschrift der Internationale Recherchenbehörde

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## C.(Fortsetzung) ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
A	EP,A,0 489 332 (BOEHRINGER INGELHEIM KG ;BOEHRINGER INGELHEIM INT (DE)) 10.Juni 1992 siehe Ansprüche ---	1-36
A	GB,A,2 059 991 (PHARMACIA FINE CHEMICALS AB) 29.April 1981 siehe das ganze Dokument -----	1-5

# INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen

PCT/EP 95/04164

## Feld I Bemerkungen zu den Ansprüchen, die sich als nicht recherchierbar erwiesen haben (Fortsetzung von Punkt 1 auf Blatt 1)

Gemäß Artikel 17(2)a) wurde aus folgenden Gründen für bestimmte Ansprüche kein Recherchenbericht erstellt:

1. ☒ Ansprüche Nr.   
 weil sie sich auf Gegenstände beziehen, zu deren Recherche die Behörde nicht verpflichtet ist, nämlich   
 Remark : Although claims 1-18,30-32 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Ansprüche Nr.   
 weil sie sich auf Teile der internationalen Anmeldung beziehen, die den vorgeschriebenen Anforderungen so wenig entsprechen, daß eine sinnvolle internationale Recherche nicht durchgeführt werden kann, nämlich
3. ☐ Ansprüche Nr.   
 weil es sich dabei um abhängige Ansprüche handelt, die nicht entsprechend Satz 2 und 3 der Regel 6.4 a) abgefaßt sind.

## Feld II Bemerkungen bei mangelnder Einheitlichkeit der Erfindung (Fortsetzung von Punkt 2 auf Blatt 1)

Die internationale Recherchenbehörde hat festgestellt, daß diese internationale Anmeldung mehrere Erfindungen enthält:

1. ☐ Da der Anmelder alle erforderlichen zusätzlichen Recherchegebühren rechtzeitig entrichtet hat, erstreckt sich dieser internationale Recherchenbericht auf alle recherchierbaren Ansprüche der internationalen Anmeldung.
2. ☐ Da für alle recherchierbaren Ansprüche die Recherche ohne einen Arbeitsaufwand durchgeführt werden konnte, der eine zusätzliche Recherchegebühr gerechtfertigt hätte, hat die Internationale Recherchenbehörde nicht zur Zahlung einer solchen Gebühr aufgefordert.
3. ☐ Da der Anmelder nur einige der erforderlichen zusätzlichen Recherchegebühren rechtzeitig entrichtet hat, erstreckt sich dieser internationale Recherchenbericht nur auf die Ansprüche der internationalen Anmeldung, für die Gebühren entrichtet worden sind, nämlich auf die Ansprüche Nr.
4. ☐ Der Anmelder hat die erforderlichen zusätzlichen Recherchegebühren nicht rechtzeitig entrichtet. Der internationale Recherchenbericht beschränkt sich daher auf die in den Ansprüchen zuerst erwähnte Erfindung; diese ist in folgenden Ansprüchen erfaßt:

Bemerkungen hinsichtlich eines Widerspruchs

- ☐ Die zusätzlichen Gebühren wurden vom Anmelder unter Widerspruch gezahlt.
- ☐ Die Zahlung zusätzlicher Gebühren erfolgte ohne Widerspruch.

# INTERNATIONALER RECHERCHENBERICHT

Angaben zu Veröffentlichungen, die zur selben Patentfamilie gehören

Internationales Aktenzeichen

PC1/EP 95/04164

Im Recherchenbericht angeführtes Patentdokument	Datum der Veröffentlichung	Mitglied(er) der Patentfamilie	Datum der Veröffentlichung
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